

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/NL91/00171        (22) International Filing Date: 13 September 1991 (13.09.91)        (30) Priority data:        90202434.8 13 September 1990 (13.09.90) EP        (34) Countries for which the regional or international application was filed: AT et al.</p> <p>(71) Applicants (for all designated States except US): GIST-BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL).</p> <p>(72) Inventors; and        (75) Inventors/Applicants (for US only) : VAN DEN ELZEN, Peter, J., M. [NL/NL]; Cayennehof 26, NL-2215 BH Voorhout (NL). PEN, Jan [NL/NL]; Voszegge 9, NL-2318 ZJ Leiden (NL). HOEKEMA, Andreas [NL/NL]; Warmonderweg 66, NL-2341 KX Oegstgeest (NL). SIJMONS, Peter, Christiaan [NL/NL]; Valeriusstraat 210-3, NL-1075 GK Amsterdam (NL). VAN OYEN, Albert, J., J. [NL/NL]; Overburgkade 78, NL-2275 XX Voorburg (NL). RIETVELD, Krijn [NL/NL]; van der Waalsstraat 129, NL-3132 TL Vlaardingen (NL). QUAX, Wilhelmus, Johannes [NL/NL]; Jan van Galenlaan 8, NL-2253 VB Voorschoten (NL).</p>		<p>(74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents and Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).</p> <p>(81) Designated States: AU, CA, JP, US.</p> <p>Published With international search report.</p>	
<p>(54) Title: TRANSGENIC PLANTS HAVING A MODIFIED CARBOHYDRATE CONTENT</p>			
<p>(57) Abstract</p> <p>The present invention provides plants with a modified taste, solids content and/or texture. The invention also provides methods of obtaining such plants via transformation with DNA constructs containing genes encoding enzymes capable of degrading plant polysaccharides and optionally additional genes encoding enzymes which are capable of further modifying the degradation products resulting from the first degradation step.</p>			

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5     Transgenic Plants Having a Modified Carbohydrate Content

## Field of the Invention

The present invention relates to the development of transgenic plants having a modified carbohydrate composition.

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## Background of the Invention

It has long been an objective of the agriculture industry to develop crops having a modified carbohydrate composition, thus providing plants or plant organs more suitable for 15 certain applications. Such modified crops provide plant products having a modified flavor, a higher content of desired saccharides and/or a more desirable texture. These crops may be either consumed directly or used in further processing.

20     In several plant species such as corn (Shannon & Garwood, 1984), pea (Bhattacharyya *et al.*, 1990), potato (Hovenkamp-Hermelink *et al.*, 1987), *Arabidopsis* (Caspar *et al.*, 1985; Lin *et al.*, 1988a; Lin *et al.*, 1988b) and tobacco (Hanson *et al.*, 1988), mutants with an altered carbohydrate composition 25 have been found. This phenomenon may be attributable to mutations found mainly in enzymes involved in the regulation of the synthesis of starch. Some of these mutants are already used in the food industry, such as sweet corn (Shannon & Garwood, *supra*), which may be directly consumed.

30     Mutants altered in starch metabolism may be obtained via classical techniques such as random screening procedures and breeding. However, these methods are laborious and time consuming processes. Moreover, breeding may give rise to the phenotype that is screened for, but may lead to the loss of 35 other desired characteristics, or the introduction of highly undesired characteristics (such as potatoes having a high alkaloid content). Changing plant characteristics through genetic engineering is a precise and predictable method, the nature of the gene which is spliced into the genome is known 40 and no undesired genes are integrated simultaneously.

Finally, modification of a specific characteristic, for instance, the alteration of the level or nature of certain products in the mutant is often difficult or even impossible using classical techniques. As such, genetic modification 5 techniques have opened up new strategies and lead to new products that cannot be obtained by classical techniques.

It would be clearly advantageous to develop sophisticated and predictable methods for obtaining plants having a modified carbohydrate composition, based on genetical 10 engineering techniques.

In U.S. Patent 4,801,540, DNA fragments are disclosed encoding an enzyme capable of hydrolyzing poly (1,4- $\alpha$ -D galacturonide) glycan into galacturonic acid. Expression constructs are provided in which the structural gene encoding 15 this enzyme is linked to modified regulatory regions in order to modulate the expression of the enzyme. The purpose of the invention as disclosed in the publication is to decrease expression levels of the polygalacturonase enzyme in order to inhibit the degradation of polygalacturonic acid and thus 20 control fruit ripening.

In PCT application WO 89/12386, plants and methods are disclosed in which the carbohydrate content is modified through the expression, in planta, of enzymes such as sucrase and levan sucrase. The object of the invention is to increase 25 the concentration of high molecular weight carbohydrate polymers in fruit in order to alter soluble solids and viscosity.

European Patent Application 438,904 describes the modification of plant metabolism (especially in tubers) 30 whereby the level of phosphofructokinase activity is increased, resulting in significantly reduced levels of sucrose and reducing sugars accumulating in the tubers.

PCT application WO 90/12876 describes the regulation of endogenous  $\alpha$ -amylase activity in genetically modified potato 35 plants. The disclosure states that a reduction of potato  $\alpha$ -amylase activity, and thus a reduction of the degradation of starch to reducing sugars is desirable for the production of potato chips as reducing sugars may be subjected to Maillard reactions during the frying of the potatoes which leads to a

detrimental effect on the flavor and texture of the product. On the other hand, the disclosure states that a higher potato  $\alpha$ -amylase activity, and thus a higher reducing sugar content is desired if the modified potato tubers are to be used for 5 fermentation for the production of spirits.

#### Summary of the Invention

The present invention provides transgenic plants or plant organs which have a modified polysaccharide composition, as 10 well as methods for the production of such plants. This is achieved via the introduction into the plant of a DNA sequence encoding an enzyme which is capable of degrading plant polysaccharides.

The present invention also provides DNA expression 15 constructs and vectors for the transformation of plants. The expression constructs are under the control of regulatory sequences which are capable of directing the expression of the selected polysaccharide modification enzymes. These regulatory sequences may also include sequences capable of 20 directing the expression of the chosen enzymes at a desired developmental stage of the plant or plant organ and/or tissue specifically.

Furthermore, depending on the products desired in planta, one or more additional expression constructs may be 25 introduced into the plant. These additional expression constructs contain DNA sequences encoding secondary enzymes which convert the degradation products resulting from the first enzymatic reaction to the desired oligo- or monosaccharides.

30 The transgenic plants provided by the present invention find applications as new products with a modified taste, solids content and/or more desirable texture.

#### Brief Description of the Figures

35 Figure 1. Binary vector pMOG23.  
Figure 2. Genomic sequence of the  $\alpha$ -amylase gene of Bacillus licheniformis as present in the vector pPROM54.  
Figure 3. Synthetic oligonucleotide duplexes used for the

various constructions.

Figure 4. Binary plasmid pMOG228, which comprises binary vector pMOG23 containing the genomic DNA sequence encoding mature  $\alpha$ -amylase from Bacillus licheniformis preceded by a methionine translation initiation codon.

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Figure 5. Binary plasmid pMOG450, which comprises binary vector pMOG23 containing the genomic DNA sequence encoding mature  $\alpha$ -amylase from Bacillus licheniformis, preceded by a methionine translation initiation codon and under the control of the class-I patatin promoter from potato.

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Figure 6. Binary plasmid pMOG437, which comprises binary vector pMOG23 containing DNA sequences encoding mature  $\alpha$ -amylase from Bacillus licheniformis and mature glucoamylase from Aspergillus niger, both preceded by a methionine translation initiation codon and both under the control of 15 a class-I patatin promoter from potato.

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#### Detailed Description of the Preferred Embodiments

The present invention provides transgenic plants or plant organs which have a modified polysaccharide composition and 25 overcomes the disadvantages encountered in classical plant breeding techniques by the stable introduction into the plants of DNA sequences encoding certain enzymes which are capable of polysaccharide degradation.

It was found unexpectedly that the transformation of 30 tobacco with a bacterial  $\alpha$ -amylase gene (lacking a secretory signal sequence) resulted in the accumulation of maltodextrins such as maltose and maltotriose, which is indicative of  $\alpha$ -amylase activity. This finding demonstrates that it is possible to modify polysaccharide composition in 35 planta by the introduction and translation of a gene encoding a polysaccharide degrading enzyme.

The observed degradation of starch by the introduction of the  $\alpha$ -amylase enzyme is very surprising since in plant cells, the entire process of starch synthesis occurs in the specific

organelles (chloroplasts, amyloplasts and the like) where starch is stored, whereas the expressed  $\alpha$ -amylase is expected to be present in the cytoplasm since no sequences were present to direct the  $\alpha$ -amylase to these organelles. Certain 5 starch degrading enzymes are endogenous to the cytoplasm of plant leaf cells. However, their function in the cytoplasm has never been conclusively explained and has never been correlated with the degradation of starch, in planta, because of the compartmental division of the two entities (Caspar et 10 al., 1989; Lin et al., 1988 a,b and c; Okita et al., 1979).

According to the present invention, the extent to which the taste and/or texture of the plants is modified may be regulated using a variety of means including the choice of the 15 regulatory regions of the DNA construct designed for the expression of the enzyme of interest and the targeting of the expressed enzyme to a pre-determined intracellular locus.

The choice of the enzyme or enzymes of interest is clearly of paramount importance in obtaining the desired 20 final product. Should more than one enzyme of interest be expressed in a plant, the ratios of the respective enzymes may be chosen in order to obtain the optimal effect (e.g. the desired sweetness).

The regulation of the expression of the enzyme(s) of 25 interest with respect to expression level and spatial (tissue/organ specific) and/or developmental regulation of expression is also a means of obtaining an optimal product. For example, the type and strength of the promoter with respect to the timing and/or location of the expression of 30 the enzyme(s) of interest will provide optimal levels of the enzyme(s) of interest in the desired locus of the transformed plant.

Finally, the locus (e.g. cellular compartment or organelle) to which the expressed enzyme may be targeted can 35 be chosen so that an optimal effect, such as better access to the substrate, is obtained.

Variations in expression levels are sometimes observed as a result of varying copy number and/or site of integration of the transforming DNA. This natural variation may be used to

select those individual plants from the pool of transgenic plants which have the desired characteristics in terms of sweetness, texture and the like. These individual plants can be used for multiplication and/or breeding with other 5 varieties.

Combinations of the above measures may also be used to obtain the desired effect. Methods of obtaining optimal products may be determined by the skilled artisan using the teaching found below.

10 According to the present invention, (primary) enzymes of interest to be expressed in plants include any enzymes or combination of enzymes which are capable of degrading plant polysaccharides. Especially preferred are enzymes encoded by DNA sequences which are of microbial origin. If necessary, 15 the coding and/or regulatory sequences may be modified to achieve cytoplasmic or organellar expression, tissue specificity or expression at a desired maturity stage of the plant or plant organ. Furthermore, codons may be modified to improve expression of the gene in the selected plant host.

20 Enzymes of interest capable of use in conjunction with the present invention include:

- a) starch degrading enzymes such as 1)  $\alpha$ -amylases (EC 3.2.1.1); 2) exo-1,4- $\alpha$ -D glucanases such as amyloglucosidases (EC 3.2.1.3),  $\beta$ -amylases (EC 3.2.1.2), 25  $\alpha$ -glucosidases (EC 3.2.1.20), and other exo-amylases; and 3) starch debranching enzymes, such as isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and the like;
- b) cellulases such as exo-1,4- $\beta$ -cellobiohydrolase (EC 3.2.1.91), exo-1,3- $\beta$ -D-glucanase (EC 3.2.1.39),  $\beta$ -glucosidase (EC 3.2.1.21) and the like;
- c) endoglucanases such as endo-1,3- $\beta$ -glucanase (EC 3.2.1.6) and endo-1,4- $\beta$ -glucanase (EC 3.2.1.4) and the like;
- d) L-arabinases, such as endo-1,5- $\alpha$ -L-arabinase (EC 3.2.1.99),  $\alpha$ -arabinosidases (EC 3.2.1.55) and the like;
- e) galactanases such as endo-1,4- $\beta$ -D-galactanase (EC 3.2.1.89), endo-1,3- $\beta$ -D-galactanase (EC 3.2.1.90),  $\alpha$ -galactosidase (EC 3.2.1.22),  $\beta$ -galactosidase (EC 3.2.1.23) and the like;
- f) mannanases, such as endo-1,4- $\beta$ -D-mannanase (EC 3.2.1.78),

$\beta$ -mannosidase (EC 3.2.1.25),  $\alpha$ -mannosidase (EC 3.2.1.24) and the like;

g) xylanases, such as endo-1,4- $\beta$ -xylanase (EC 3.2.1.8),  $\beta$ -D-xylosidase (EC 3.2.1.37), 1,3- $\beta$ -D-xylanase, and the like;

5 h) other enzymes such as  $\alpha$ -L-fucosidase (EC 3.2.1.51),  $\alpha$ -L-rhamnosidase (EC 3.2.1.40), levanase (EC 3.2.1.65), inulanase (EC 3.2.1.7), and the like.

Optionally, in a further embodiment, the present invention also contemplates the introduction to the target 10 (host) plant of one or more additional DNA constructs encoding secondary enzymes of interest which are capable of further modifying the polysaccharide degradation products (obtained from the action of the primary polysaccharide degrading enzyme(s)) to desired saccharide subunits. 15 Especially preferred secondary enzymes are enzymes encoded by DNA sequences which are of microbial origin.

To illustrate, secondary enzymes of particular interest, which are capable of further degrading the maltose, maltotriose and  $\alpha$ -dextrins obtained from the first 20 degradation of starch, include inter alia, maltases,  $\alpha$ -dextrinase,  $\alpha$ -1,6-glucosidases, and the like. The action of these enzymes result in the formation of glucose.

In yet a further embodiment of the present invention, if desired, one or more further secondary enzymes, which are 25 capable of modifying monosaccharides, may be expressed in the same plant. Such enzymes include but are not limited to glucose isomerase, invertase, and the like.

The source from which DNA sequences encoding these enzymes of interest may be obtained is not relevant, provided 30 the enzyme is active in the environment in which the enzyme is expressed or in which the expressed enzyme is targeted. The choice of both the primary (plant polysaccharide degrading) and, if desired, secondary enzymes of interest may depend on the substrate specificity and/or the desired 35 saccharide end-product.

The enzymes of interest may be expressed constitutively in the transgenic plants during all stages of development. Depending on the use of the plant or plant organs, the enzymes may be expressed in a stage-specific manner, for

instance during tuber formation or fruit development. Furthermore, depending on the use, the enzymes may be expressed tissue-specifically, for instance in plant organs such as fruit, tubers, leaves or seeds.

5 Plant polysaccharides, as defined within the context of the present invention are intended to consist of polyhydroxy aldehydes or ketones, consisting of more than six covalently-linked monosaccharides, which are normally found in plants prior to the action of the enzyme or enzymes of interest 10 according to the present invention. Such polysaccharides are typically polymers of D-arabinose, D-fructose, D- and L-galactose, D-glucose, and D-xylose and mannose.

Saccharide subunits, the desired end-products of the present invention, are defined as saccharides having a 15 shorter chain length than the original polysaccharide, including monosaccharides, which are obtained via the action of one or more enzymes of interest on the plant polysaccharides.

Transgenic plants, as defined in the context of the 20 present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of at least one enzyme of interest in the desired plant or plant organ.

25 Plants capable of being used in conjunction with the present invention include, but are not limited to crops producing edible flowers such as cauliflower (Brassica oleracea), artichoke (Cynara scolymus), fruits such as apple (Malus, e.g. domesticus), banana (Musa, e.g. acuminata), 30 berries (such as the currant, Ribes, e.g. rubrum), cherries (such as the sweet cherry, Prunus, e.g. avium), cucumber (Cucumis, e.g. sativus), grape (Vitis, e.g. vinifera), lemon (Citrus limon), melon (Cucumis melo), nuts (such as the walnut, Juglans, e.g. regia; peanut, Arachis hypogaeae), 35 orange (Citrus, e.g. maxima), peach (Prunus, e.g. persica), pear (Pyra, e.g. communis), pepper (Solanum, e.g. capsicum), plum (Prunus, e.g. domestica), strawberry (Fragaria, e.g. moschata), tomato (Lycopersicon, e.g. esculentum), leafs, such as alfalfa (Medicago, e.g. sativa), cabbages (such as

Brassica oleracea), endive (Cichoreum, e.g. endivia), leek (Allium, e.g. porrum), lettuce (Lactuca, e.g. sativa), spinach (Spinacia e.g. oleraceae), tobacco (Nicotiana, e.g. tabacum), roots, such as arrowroot (Maranta, e.g. arundinacea), beet (Beta, e.g. vulgaris), carrot (Daucus, e.g. carota), cassava (Manihot, e.g. esculenta), turnip (Brassica, e.g. rapa), radish (Raphanus, e.g. sativus), yam (Dioscorea, e.g. esculenta), sweet potato (Ipomoea batatas) and seeds, such as bean (Phaseolus, e.g. vulgaris), pea (Pisum, e.g. sativum), soybean (Glycin, e.g. max), wheat (Triticum, e.g. aestivum), barley (Hordeum, e.g. vulgare), corn (Zea, e.g. mays), rice (Oryza, e.g. sativa), tubers, such as kohlrabi (Brassica, e.g. oleraceae), potato (Solanum, e.g. tuberosum), and the like.

15 The choice of the plant species is determined by the intended use of the plant or parts thereof and the amenability of the plant species to transformation.

20 The expression of recombinant genes in plants involves such details as transcription of the gene by plant polymerases, translation of mRNA, etc., which are known to persons skilled in the art of recombinant DNA techniques. Only details relevant for the proper understanding of this invention are discussed below.

25 Regulatory sequences which are known or are found to cause expression of a gene encoding an enzyme of interest in planta may be used in the present invention. The choice of the regulatory sequences used depends on the target crop and/or target organ of interest. Such regulatory sequences may be obtained from plants or plant viruses, or may be 30 chemically synthesized. Such regulatory sequences are promoters active in directing transcription in plants, either constitutively or developmental stage- and/or tissue-specifically, depending on the use of the plant or parts thereof. These promoters include, but are not limited to 35 promoters showing constitutive expression, such as the 35S promoter of Cauliflower Mosaic Virus (CaMV) (Guilley et al., 1982), those for leaf-specific expression, such as the promoter of the ribulose bisphosphate carboxylase small subunit gene (Coruzzi et al., 1984), those for root-specific

expression, such as the promoter from the glutamine synthase gene (Tingey *et al.*, 1987), those for seed-specific expression, such as the cruciferin A promoter from Brassica napus (Ryan *et al.*, 1989), those for tuber-specific 5 expression, such as the class-I patatin promoter from potato (Rocha-Sosa *et al.*, 1989; Wenzler *et al.*, 1989) or those for fruit-specific expression, such as the polygalacturonase (PG) promoter from tomato (Bird *et al.*, 1988).

Other regulatory sequences such as terminator sequences 10 and polyadenylation signals include any such sequence functioning as such in plants, the choice of which is within the level of the skilled artisan. An example of such sequences is the 3' flanking region of the nopaline synthase (nos) gene of Agrobacterium tumefaciens (Bevan, 1984).

15 The regulatory sequences may also include enhancer sequences, such as found in the 35S promoter of CaMV, and mRNA stabilizing sequences such as the leader sequence of Alfalfa Mosaic Virus (AlMV) RNA4 (Brederode *et al.*, 1980) or any other sequences functioning in a like manner.

20 In one embodiment of the present invention, if simple expression of an enzyme of interest into the cytoplasm of the plant cell should be desired, the expressed enzyme should not contain a secretory signal peptide or any other targeting sequence.

25 In another embodiment of the present invention, the DNA construct encoding a selected enzyme of interest according to the present invention may optionally be provided with leader sequences capable of targeting the expressed enzyme to a pre-determined locus in order to have better access of the enzyme 30 to its substrate. Targeting sequences which may be operably coupled to the enzyme of interest in order to achieve this function have been described in the literature (Smeekens *et al.*, 1990; van den Broeck *et al.*, 1985; Schreier *et al.*, 1985). For example, to obtain expression in chloroplasts and 35 mitochondria, the expressed enzyme should contain a specific so-called transit peptide for import into these organelles (Smeekens *et al.*, 1990). If the activity of the enzyme is desired in the vacuoles, a secretory signal sequence must be present, as well as a specific targeting sequence that

directs the enzyme to these vacuoles (Tague *et al.*, 1988). This may also lead to the targeting of the enzyme to seeds.

All parts of the relevant DNA constructs (promoters, regulatory-, stabilizing-, targeting- or termination sequences) of the present invention may be modified, if desired, to affect their control characteristics using methods known to those skilled in the art.

Several techniques are available for the introduction of the expression construct containing a DNA sequence encoding an enzyme of interest into the target plants. Such techniques include but are not limited to transformation of protoplasts using the calcium/polyethylene glycol method, electroporation and microinjection or (coated) particle bombardment (Potrykus, 1990).

In addition to these so-called direct DNA transformation methods, transformation systems involving vectors are widely available, such as viral vectors (e.g. from the Cauliflower Mosaic Virus (CaMV), Fraley *et al.*, 1986) and bacterial vectors (e.g. from the genus Agrobacterium) (Potrykus, 1990). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using methods known in the art (Horsch, *et al.*, 1985). The choice of the transformation and/or regeneration techniques is not critical for this invention.

For dicots, an embodiment of the present invention employs the principle of the binary vector system (Hoekema *et al.*, 1983; Schilperoort *et al.*, 1984) in which Agrobacterium strains are used which contain a vir plasmid with the virulence genes and a compatible plasmid containing the gene construct to be transferred. This vector can replicate in both E.coli and in Agrobacterium, and is derived from the binary vector Bin19 (Bevan, 1984) which is altered in details that are not relevant for this invention. The binary vectors as used in this example contain between the left- and right-border sequences of the T-DNA, an identical NPTII-gene coding for kanamycin resistance (Bevan, 1984) and a multiple cloning site to clone in the required gene constructs.

The transformation and regeneration of monocotyledonous crops is not a standard procedure. However, recent scientific

progress shows that in principle monocots are amenable to transformation and that fertile transgenic plants can be regenerated from transformed cells. The development of reproducible tissue culture systems for these crops, together with the powerful methods for introduction of genetic material into plant cells has facilitated transformation. Presently the methods of choice for transformation of monocots are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation of protoplasts. For example, transgenic rice plants have been successfully obtained using the bacterial hph gene, encoding hygromycin resistance, as a selection marker. The gene was introduced by electroporation (Shimamoto et al., 1989). Transgenic maize plants have been obtained by introducing the bar gene from Streptomyces hygroscopicus, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microparticle bombardment (Gordon-Kamm et al., 1990). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee et al., 1989). The stable transformation of wheat cell suspension cultures via microprojectile bombardment has recently been described (Vasil et al., 1991). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil et al., 1990). The combination of regeneration techniques with transformation systems for these crops enables the application of the present invention to monocots. These methods may also be applied for the transformation and regeneration of dicots.

If desired, a number of methods may be used to obtain transgenic plants in which more than one enzyme of interest is expressed. These include but are not limited to:

- a. Cross-fertilization of transgenic plants each expressing a different enzyme of interest.
- b. Plant transformation with a DNA fragment or plasmid that contains multiple genes, each encoding an enzyme of

interest, each containing its own necessary regulatory sequences.

- c. Plant transformation with different DNA fragments or plasmids simultaneously, each containing a gene for an enzyme of interest, using the necessary regulatory sequences.
- 5 d. Successive transformations of plants, each time using a DNA fragment or plasmid encoding a different enzyme of interest under the control of the necessary regulatory sequences.
- 10 e. A combination of the methods mentioned above.

The choice of the above methods is not critical with respect to the objective of this invention.

In one embodiment of the present invention, an  $\alpha$ -amylase 15 is constitutively expressed intracellularly in tobacco and tomato plants, resulting in the degradation of starch in these plants to lower molecular weight saccharides. A genomic DNA fragment encoding mature  $\alpha$ -amylase from Bacillus licheniformis, i.e. encoding the  $\alpha$ -amylase without the signal 20 peptide sequence, is placed under the control of the CaMV 35S promoter and enhancer sequences. The mRNA stabilizing leader sequence of RNA4 from AlMV is included, as well as the terminator and polyadenylation signal sequences of the nopaline synthase (nos) gene of Agrobacterium tumefaciens. 25 The construct is thereafter subcloned into a binary vector such as pMOG23 (deposited at the Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands on January 29, 1990 under accession number CBS 102.90). This vector is introduced into Agrobacterium tumefaciens which contains a disarmed Ti- 30 plasmid. Bacterial cells containing this construct are co-cultivated with tissues from the target plants, and transformed plant cells are selected on nutrient media containing antibiotics and induced to regenerate into differentiated plants on such media. The resulting plants 35 contain the stably integrated gene and express the  $\alpha$ -amylase intracellularly.

The  $\alpha$ -amylase enzyme activity of the transgenic plants may be tested with direct enzyme assays using colorimetric techniques or gel assays. The assay of choice is not critical

to the present invention. The protein is detectable on Western blots with antibodies raised against  $\alpha$ -amylase from Bacillus licheniformis.

The plants may be qualitatively assayed for starch 5 content either by staining for starch with iodine. Plants may be quantitatively assayed for the presence of starch degradation products by using techniques as NMR and HPLC. Other methods may also be used. The choice of the method is not critical to the present invention.

10 In another preferred embodiment, both an  $\alpha$ -amylase and a glucoamylase are expressed in potatoes. The enzymes are expressed only in the tubers of the plants. The result is the degradation of starch in tubers by both enzymes to lower molecular weight saccharides. A genomic DNA fragment encoding 15 mature  $\alpha$ -amylase from Bacillus licheniformis and a cDNA fragment encoding mature glucoamylase from Aspergillus niger are each placed under the control of the tuber-specific promoter from a class-I patatin gene from potato. Both constructs also include the terminator and polyadenylation 20 signal sequences of the nopaline synthase (nos) gene of Agrobacterium tumefaciens. Both constructs are thereafter subcloned together into the binary vector pMOG23. This vector is introduced into Agrobacterium tumefaciens, which contains a disarmed Ti plasmid. Bacterial cells containing this 25 construct are cocultivated with tissues from potato plants and transformed plant cells are selected on nutrient media containing antibiotics, and induced to regenerate into differentiated plants on such media. The resulting plants contain the stably integrated genes. Both  $\alpha$ -amylase and 30 glucoamylase are expressed only in the tubers of the transformed potatoes. Both enzymes are expressed intracellularly.

The  $\alpha$ -amylase and glucoamylase enzyme activities in the transgenic tubers can be tested with various assays. For 35 example, glucoamylase activity may be determined by an assay measuring p-nitrophenol released from p-nitrophenol- $\alpha$ -D-glucopyranoside by the glucoamylase. Alpha-amylase activity may be measured as described above and in the examples provided below. The presence of both enzymes may be

demonstrated by immunoblotting, for example. The choice of assays is not relevant to the present invention.

The transgenic potato tubers may be assayed for their carbohydrate composition by using techniques for the 5 detection of sugars such as HPLC and NMR. Other methods may also be used. The choice of the method is not critical to the present invention.

Transgenic plants or plant organs (such as flowers, fruits, leaves, roots, tubers) having a higher content of 10 polysaccharide degradation products and consequently a modified flavor and/or a desired texture, may be used as a new product either as such or in a form obtained after non-fermentative processing which retains the distinctive qualities resulting from the modification of the plant 15 saccharides. Examples of such uses are the production of baby foods, juices, sauces, pastes, concentrates, sweeteners, jams, jellies, syrups, and animal feeds. Grains having an altered carbohydrate composition may be used in the productions of baked products, for example, which have a 20 modified taste. Tobaccos having an altered carbohydrate composition exhibit a modified taste and aroma.

Alternatively, the polysaccharide degradation products may be extracted from the plant or plant organs and used as such, for instance as a sweetener, or in various processes.

25 The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy 30 with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Centrigrade and pressure is at or near atmospheric.

Construction of the binary vector pMOG23.

The binary vector pMOG23 (deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, on January 29, 1990, under accession number CBS 102.90; shown in

Figure 1) is a derivative of vector Bin19 (Bevan, 1984). First, the positions of the left border (LB) and the right border (RB) were interchanged with reference to the neomycin phosphotransferase gene II (NPTII gene). Secondly, the 5 orientation of the NPTII gene was reversed giving transcription in the direction of LB. Finally, the polylinker of Bin19 was replaced by a polylinker having the following restriction enzyme recognition sites: EcoRI, KpnI, SmaI, BamHI, XbaI, SacI, XhoI, and HindIII.

10

#### Example 2

##### Cloning of the $\alpha$ -amylase gene of *Bacillus licheniformis*

All transformations in this example were performed in E. coli strain DH5 $\alpha$ .

15

##### a. Tailoring of the $\alpha$ -amylase gene of *Bacillus licheniformis*

The  $\alpha$ -amylase gene (Figure 2) from Bacillus licheniformis is present in the Bacillus vector pPROM54, which is described in European Patent Application 224,294, the disclosure of 20 which is hereby incorporated by reference. The plasmid pPROM54 has been deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on November 5, 1985, under accession number CBS 696.85.

The plasmid pPROM54 was digested with XbaI and BclI. The 25 XbaI/BclI fragment was cloned in plasmid pUC18 digested with XbaI and BamHI, resulting in plasmid pMOG318. A SalI/BamHI fragment was synthesized with pMOG318 as a template with PCR technology, creating the BamHI site by use of a mismatch primer (the position of the created BamHI site is indicated 30 in Figure 2). The SalI/BamHI PCR fragment was cloned in plasmid pIC-19R (Marsh *et al.*, 1984) digested with SalI and BamHI, resulting in plasmid pMOG319. The SalI fragment from pMOG318 (the second SalI site is present in pUC18), containing the 5' end of the  $\alpha$ -amylase gene, was cloned in 35 pMOG319 digested with SalI. This resulted in plasmid pMOG320 which contains the entire  $\alpha$ -amylase gene.

##### b. Construction of vector pMOG18.

The expression cassette of pROK1 (Baulcombe *et al.*, 1986)

was cloned as an EcoRI/HindIII fragment into pUC18. This cassette contains the 800 bp Cauliflower Mosaic Virus (CaMV) 35S promoter fragment on an EcoRI/BamHI fragment and the nopaline synthase (nos) transcription terminator of 5 Agrobacterium tumefaciens on a BamHI/HindIII fragment. The promoter fragment consists of the sequence from -800 to +1 (both inclusive) of the CaMV promoter. Position +1 is the transcription initiation site (Guilley *et al.*, 1982). The sequence upstream of the NcoI site at position -512 was 10 deleted and this site was changed into an EcoRI Site. This was achieved by cutting the expression cassette present in pUC18 with NcoI, filling in the single-stranded ends with Klenow polymerase and ligation of an EcoRI linker.

The resulting plasmid was cut with EcoRI, resulting in 15 the deletion of the EcoRI fragment carrying the sequences of the CaMV 35S promoter upstream of the original NcoI site. The BamHI/HindIII fragment, containing the nos terminator was replaced by a synthetic DNA fragment (Oligonucleotide duplex A, Figure 3) containing the leader sequence of RNA4 of 20 Alfalfa Mosaic Virus (AlMV) (Brederode *et al.*, 1980). This was done by cleavage with BamHI, followed by cleavage with HindIII and ligation of the synthetic DNA fragment. The BamHI site and three upstream nucleotides were deleted by site-directed mutagenesis.

25 In the resulting plasmid, the BamHI/HindIII fragment containing the nos terminator was reintroduced. The gene encoding beta-glucuronidase (originating from plasmid pRAJ 275; Jefferson, 1987) was ligated in as an NcoI/BamHI fragment, resulting in plasmid pMOG14.

30 It is known that duplication of the sequence between -343 and -90 increases the activity of the CaMV 35S promoter (Kay *et al.*, 1987). To obtain a promoter fragment with a double, so-called enhancer sequence, the enhancer fragment from plasmid pMOG14 was isolated as an AccI/EcoRI fragment and 35 subsequently blunt-ended with Klenow polymerase. The thus-obtained fragment was introduced in pMOG14 cut with EcoRI and blunt-ended, such that the border between the blunt-ended EcoRI and AccI sites generated a new EcoRI site. The resulting plasmid pMOG18 contains the 35S CaMV promoter with

a double enhancer sequence, the leader sequence of RNA4 from AlMV and the nos terminator in an expression cassette still present as an EcoRI/HindIII fragment.

5   c. Cloning of the  $\alpha$ -amylase gene from *Bacillus licheniformis* in the binary vector.

Plasmid pMOG320 was digested with HgaI and BamHI. The HgaI/BamHI fragment was cloned together with the synthetic oligonucleotide duplex B (Figure 3) into pMOG18 digested with 10 NcoI and BamHI, resulting in plasmid pMOG322. The  $\beta$ -glucuronidase gene was thus replaced by the coding sequence for the mature  $\alpha$ -amylase of Bacillus licheniformis preceded by the ATG triplet encoding the methionine translation initiation codon. Plasmid pMOG18 contains the 35S promoter 15 and enhancer of Cauliflower mosaic virus (CaMV), the nopaline synthase (nos) terminator from Agrobacterium tumefaciens and the RNA4 leader sequence of Alfalfa mosaic virus (AlMV). The resulting construct does not contain coding information for a signal peptide. The entire construct was spliced out with 20 EcoRI and HindIII and transferred into the binary vector pMOG23 digested with EcoRI and HindIII. The resulting plasmid has been designated pMOG228 (Figure 4).

The chimeric  $\alpha$ -amylase gene on the binary plasmid pMOG228 was mobilized, in a triparental mating with the E.coli strain 25 HB101 containing plasmid pRK2013 (Ditta et al., 1980), into Agrobacterium strain LBA4404, which contains a plasmid having the virulence genes necessary for T-DNA transfer to the plant (Hoekema et al., 1983).

Example 3

Transformation of tobacco

Tobacco (Nicotiana tabacum cv. Petit Havanna SR 1) was transformed by co-cultivation of plant leaf disks (Horsch et al., 1985) with Agrobacterium tumefaciens, containing the 35 binary vector pMOG228 with the  $\alpha$ -amylase gene. Transgenic plants were selected on kanamycin resistance. The transgenic plants were assayed for activity of the enzyme of interest. Plants expressing the  $\alpha$ -amylase gene were analyzed more thoroughly and used in further experiments.

Leaf discs of about 5 x 5 mm were cut from leaves of axenically grown plants of Nicotiana tabacum cv. Petit Havanna SR1. The discs were floated for 20 minutes in MS-medium (Murashige & Skoog, 1962) containing 30 g/L sucrose with 1% (v/v) of a culture of Agrobacterium tumefaciens LBA4404 (pMOG228) ( $10^9$  cells/ml). Subsequently, the discs were briefly dried on filter paper and transferred to plates containing solid medium consisting of MS-medium, containing 30 g/L sucrose, 7 g/L agar, 1 mg/L kinetin and 0.03 mg/L naphthyl acetic acid (NAA). Two days later, the discs were transferred to plates containing the same medium plus 500 mg/L carbenicillin. After one week, the discs were again transferred to plates containing the same medium, this time with about 50 mg/L kanamycin to select for transgenic shoots. Discs were transferred to fresh plates with three week intervals. Developing shoots were excised and transferred to pots containing solid medium consisting of MS-medium, containing 30 g/L sucrose, 100 mg/L kanamycin and 100 mg/L cefotaxime for root development. After roots have developed, the plants were transferred to the soil. The plants were tested for expression of the gene of interest.

#### Example 4

##### Alpha-amylase expression in transgenic tobacco plants

Alpha-amylase activity was determined by the method described by Saito (1973) at 56 °C. Units are defined in this case as the amount of enzyme giving a reduction of the absorbance at 690 nm by 10% in 10 minutes. Specific activity for the Bacillus licheniformis  $\alpha$ -amylase was  $8.7 \times 10^5$  U/mg protein. The tip of one of the top leafs (about 100 mg) was cut off and homogenized in 100  $\mu$ l  $\alpha$ -amylase assay buffer (Saito, 1973). The homogenate was spun down for 10 minutes in an Eppendorf centrifuge. The supernatant was collected and assayed for protein and  $\alpha$ -amylase content. Control plants had levels of activity at or below the detection limit.

In the 62 transgenic plants obtained, the measured expression levels, as determined by the method of Saito (1973) varied between 0 and 3.29 U/ $\mu$ g protein. Based on the specific activity of the enzyme, these levels corresponded to

0 - 0.38 % of the total amount of soluble protein. The average was 0.11 % of the total amount of soluble protein. The protein was clearly present intracellularly, since no significant amount of  $\alpha$ -amylase activity was detected in the 5 extracellular fluid that was isolated by vacuum filtration of the leaves with buffer, followed by collection of the fluid by centrifugation (Sijmons *et al.*, 1990). These results were confirmed with immunological detection of the Bacillus licheniformis  $\alpha$ -amylase on Western blots, which demonstrated 10 that the protein is indeed the desired  $\alpha$ -amylase. Further confirmation was obtained by running extracts and extracellular fluid on polyacrylamide-SDS gels. After 15 electrophoresis, the gels were incubated in 0.04 M Tris/HCl pH 7.4 for 3 hours with 6 changes of buffer to renature the enzymes. The gels were overlaid with 0.25% potato Lintner starch, 0.75% agar in 0.05 M Tris/HCl pH 7.4 containing 1 mM CaCl<sub>2</sub>, incubated overnight at 37 °C and subsequently stained with 1mM I<sub>2</sub>/0.5 M KI in water. Alpha-amylase activity was detected as a clear zone in the overlay (Lacks & Springhorn, 20 1980). In the transgenic plants, an  $\alpha$ -amylase was detected having an apparent molecular weight of about 55,000 kDa, the same as that of the Bacillus licheniformis  $\alpha$ -amylase.

Tobacco plants expressing  $\alpha$ -amylase were pale light green (chlorotic) and somewhat retarded in growth as compared to 25 control plants.

#### Example 5

##### Carbohydrate analysis of transgenic tobacco plants

Qualitatively the starch content in transgenic tobacco 30 leaves, collected at roughly the half-way point of the photoperiod, was determined by destaining the leaves overnight by shaking in 96% ethanol, followed by staining for starch with 5.7 mM I<sub>2</sub> and 43.3 mM KI in 0.2 N HCl. Leaves containing starch stained black-blue, while leaves lacking 35 starch stained brownish-yellow (Caspar *et al.*, 1985).

Approximately 2.5 g portions of leaf material (stored in deep-freeze) obtained from control and transformed (good  $\alpha$ -amylase expressors) plants were homogenized in 10 ml water at 4 °C with an ultra-turrax. Microscopic inspection revealed

that no intact cells remained. After removal of the cell fragments by centrifugation, the glucose oligomer content in the green-colored supernatant was determined. The filtered samples were analyzed via HPLC on an Aminex HPX-42A column (300 mm x 7.8 mm, 85 °C) using water as the eluent. The presence of maltose and maltotriose were detected in the samples of the transformed plants and not in the control (untransformed) plants. The results are shown in Table 1, below.

10

Table 1

<u>Saccharides extracted from tobacco leaves and analyzed on an Aminex HPX-42A-HPLC column</u>		
<u>Preparation</u>	<u>Saccharide</u>	<u>mg Saccharide/g wet material</u>
Control	Maltotriose	undetectable
	Maltose	undetectable
Transgenic	Maltotriose	0.34
	Maltose	1.73

25

Example 6Cloning of the  $\alpha$ -amylase gene of *Bacillus licheniformis* in a tuber-specific expression construct

All transformations in *E. coli* in this example were performed in strain DH5 $\alpha$ .

30

To construct an expression cassette for tuber-specific expression, the promoter from a class-I patatin gene of potato (*Solanum tuberosum* cv. Bintje) is synthesized using PCR technology with isolated genomic DNA (Mettler, 1987) as a template. Class-I patatin genes show tuber-specific expression in potato. Both the coding and flanking sequences of several members of the patatin multigene family have been determined (Rocha-Sosa *et al.*, 1989; Bevan *et al.*, 1986; Mignery *et al.*, 1988). Chimeric genes have been reported containing 5' flanking regions of a class-I patatin gene fused to  $\beta$ -glucuronidase, giving rise to tuber-specific expression of  $\beta$ -glucuronidase (Wenzler *et al.*, 1989).

Two oligonucleotides corresponding to the sequence of the pAT21 and B33 genes (Mignery *et al.*, 1989; Bevan *et al.*,

1986), are synthesized, allowing the amplification of the class-I patatin 5' flanking region as a HindIII/NcoI fragment:

5' ATTAAAGCTTATGTTGCCATATAGAGTAGT 3'  
5 5' GTAGGATCCATGGTGCAAATGTTCAAAGTGT 3'

The oligonucleotides are designed to contain suitable restriction sites (HindIII and NcoI) at their termini to allow assembly of the expression cassette after digestion of the fragments with the restriction enzymes. A fragment of 10 about 1.3 kb containing a functional class-I patatin promoter fragment was synthesized. After addition of EcoRI synthetic linkers by ligation, the fragment was cloned in pUC18 linearized with EcoRI, resulting in plasmid pMOG546. In a three-way ligation, the HindIII/NcoI-fragment of plasmid 15 pMOG546, together with the NcoI/HindIII fragment of plasmid pMOG322 (see Example 2, encoding mature  $\alpha$ -amylase of Bacillus licheniformis preceded by an ATG translation initiation codon and followed by the nos terminator from Agrobacterium tumefaciens) were ligated into the binary vector pMOG23 cut 20 with HindIII, resulting in the binary plasmid pMOG450 (see Figure 5).

#### Example 7

##### Transformation of tomato

25 Tomato (Lycopersicon esculentum cv. Moneymaker) was transformed with the Agrobacterium strain LBA4404 (pMOG228). The basic culture medium consisted of MS-medium (Murashige & Skoog, 1962), supplemented with 30 g/L sucrose, B5 vitamins (Gamborg, 1970), 2 mg/L zeatin riboside and 0.1 mg/L indole 30 acetic (IAA). The media were solidified where necessary with 0.7 g/L Daichin agar.

Cotyledons of six day old, axenically grown seedlings were cut on both ends and pre-incubated for 24 hours on solid medium with a feeder of a 10 day old Petunia cell suspension. 35 The cotyledons were subsequently co-cultivated for 20 hours with a log-phase culture of Agrobacterium tumefaciens strain LBA4404 (pMOG228) which was washed with MS-medium. The cotyledons were dried briefly on sterile filter paper and placed on solid medium with a feeder layer of a 10 day old

Petunia cell suspension. After 48 hours, the cotyledons were transferred to plates containing the same medium without the feeder layer and with 200 mg/L cefotaxim and 100 mg/L vancomycin. Five days after co-cultivation, the cotyledons 5 were transferred to the same medium plus 100 mg/L kanamycin. The cotyledons were transferred to fresh plates every three weeks.

Shoots were excised and placed on rooting medium (MS- medium supplemented with 10 g/L sucrose, 100 mg/L cefotaxim 10 and 50 mg/L vancomycin). After rooting, the plants were transferred to the soil and subsequently tested for  $\alpha$ -amylase expression.

#### Example 8

15 Expression of  $\alpha$ -amylase from *Bacillus licheniformis* in tomato and carbohydrate analysis of the transgenic fruit

Transgenic tomato plants obtained from the transformation with the constitutive expression construct pMOG228 did not show phenotypic effects. Leaves of the transgenic tomato 20 plants grown for three weeks in soil were assayed for  $\alpha$ -amylase activity as described in Example 4. Expression levels of  $\alpha$ -amylase in the plants analyzed varied between 0 and 1.2 U/ $\mu$ g soluble protein. The presence of the enzyme was confirmed with Western blotting using antibodies raised 25 against *Bacillus licheniformis*  $\alpha$ -amylase.

The starch content in leaves obtained from plants grown for 3 weeks in soil and collected half-way through the photoperiod was determined as described in Example 5. Transgenic plants expressing  $\alpha$ -amylase contained demonstrably 30 less starch in their leaves than control plants.

#### Example 9

Cloning of a cDNA encoding mature glucoamylase from *Aspergillus niger*

35 All transformations in *E.coli* in this example were performed in strain DH5 $\alpha$ .

a. Isolation of poly A<sup>+</sup> RNA from *Aspergillus niger*

About 1 x 10<sup>8</sup> spores of *Aspergillus niger* strain DS 2975

(deposited at the Centraal Bureau voor Schimmelcultures on August 10, 1988, under number CBS 513.88) are inoculated in 100 ml pre-culture medium containing (per liter): 1 g  $\text{KH}_2\text{PO}_4$ ; 30 g maltose; 5 g yeast-extract; 10 g casein-hydrolysate; 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 3 g Tween 80. The pH is adjusted to 5.5.

After growing overnight at 34 °C in a rotary shaker, 1 ml of the growing culture is inoculated in a 100 ml main-culture containing (per liter): 2 g  $\text{KH}_2\text{PO}_4$ ; 70 g malto-dextrin (Maldex MDO<sub>3</sub>, Amylum); 12.5 g yeast-extract; 25 g casein-hydrolysate; 2 g  $\text{K}_2\text{SO}_4$ ; 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.03 g  $\text{ZnCl}_2$ ; 0.02 g  $\text{CaCl}_2$ ; 0.05 g  $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$  and  $\text{FeSO}_4$ . The pH is adjusted to 5.6. The mycelium is grown for 140 hours and harvested. 0.5 g of dry mycelium is frozen with liquid nitrogen and ground. The material is subsequently homogenized with an Ultra turrax (full speed, 1 minute) at 0 °C in 10 ml 3 M LiCl, 6 M Urea and maintained overnight at 4 °C as described by Auffray and Rougeon (1980). Total cellular RNA is obtained after centrifugation at 16,000 g and dissolved in 3 ml 10 mM Tris-HCl (pH 7.4), 0.5% SDS and extracting twice with phenol:chloroform:isoamylalcohol (50:48:2). The RNA is precipitated with ethanol and redissolved in 1 ml 10 mM Tris-HCl (pH 7.4), 0.5% SDS. For poly A<sup>+</sup> selection, the total RNA sample is heated for 5 minutes at 65 °C, adjusted to 0.5 M NaCl and subsequently applied to an oligo(dT)-cellulose column. After several washes with a solution containing 10 mM Tris pH 7.0, 0.5% SDS and 0.1 mM NaCl, the poly A<sup>+</sup> RNA is collected by elution with 10 mM Tris pH 7.0 and 0.5% SDS.

b. Preparation and cloning of a cDNA encoding glucoamylase

To synthesize the first strand of the cDNA, 5 µg of poly A<sup>+</sup> RNA, isolated according to Example 11a, is dissolved in 16.5 µl H<sub>2</sub>O and the following components are added: 2.5 µl RNasin (30 U/µl), 10 µl of a buffer containing 50 mM Tris, 6 mM MgCl<sub>2</sub> and 40 mM KCl, 2 µl 1 M KCl, 5 µl 0.1 M DTT, 0.5 µl oligo(dT)<sub>12-18</sub> (2.5 mg/ml), 5 µl 8 mM dNTP-mix, 5 µl BSA (1 mg/ml) and 2.5 µl Moloney MLV reverse transcriptase (200 U/µl). The mixture is incubated for 30 minutes at 37 °C and the reaction is stopped by adding 10 µl 0.2 M EDTA and 50 µl H<sub>2</sub>O. An extraction is performed using 110 µl chloroform and

following centrifugation for 5 minutes, the aqueous layer is collected and 110  $\mu$ l 5 M NH<sub>4</sub>Ac and 440  $\mu$ l absolute ethanol (temperature: -20°C) are added. Precipitation is performed in a dry ice/ethanol solution for 30 minutes. Following 5 centrifugation for 10 minutes at 0°C, the cDNA/mRNA pellet is washed with 70% ice-cold ethanol. The pellet is dried and dissolved in 20  $\mu$ l of H<sub>2</sub>O.

Isolation of a cDNA encoding glucoamylase is performed with the Polymerase Chain Reaction. Two oligonucleotides are 10 synthesized, based on the nucleotide sequence of glucoamylase G1 cDNA published by Boel *et al.* (1984).

Oligo 1 : 5' CTTCCACCATGGCGACCTTGGATTC 3'

Oligo 2 : 5' AGCTCGAGCTCACCGCCAGGTGTC 3'

With these two oligonucleotides, the region encoding the 15 mature enzyme, i.e. without secretory signal peptide and pro-peptide, preceded by a translation initiation ATG codon (underlined) and flanked by suitable cloning sites is amplified. The obtained DNA is digested with NcoI and SstI. Together with the SstI/HindIII-fragment of p35SGUSINT 20 (Vancanneyt *et al.*, 1990) containing the terminator transcript fragment of the CaMV 35S, the NcoI/SstI fragment is cloned in a three-way ligation into pMOG18 (see Example 2), which is digested with NcoI and HindIII, resulting in plasmid pMOG567.

25 The PstI/SstI-fragment of pMOG567 is subsequently cloned in pIC20H (Marsh *et al.*, 1984), digested with PstI and SstI. In the resulting plasmid, the PstI/HindIII-fragment is replaced by the corresponding amyloglucosidase cDNA-fragment, resulting in pMOG568. The sequence of the HindIII/SstI 30 fragment is compared to the sequence published by Boel *et al.* (1984). The PstI/SstI-fragment of pMOG568 is ligated to the PstI/StyI-fragment of the amyloglucosidase cDNA, and the resulting fragment is cloned in a three-way ligation, together with a synthetic adaptor:

35 5' CATGGCGAC 3'

3' CGCTGGAAC 5'

into pMOG567 digested with NcoI and SstI, resulting in plasmid pMOG569 which encodes mature amyloglucosidase under control of the CaMV 35S promoter and terminator.

Example 10Cloning of both  $\alpha$ -amylase from *Bacillus licheniformis* and glucoamylase from *Aspergillus niger*

5 All transformations in this example are performed in *E. coli* strain DH5 $\alpha$ .

10 The HindIII/NcoI class-I patatin promoter fragment (see Example 6) from plasmid pMOG546 is cloned, together with the NcoI/HindIII fragment of plasmid pMOG567 encoding mature amyloglucosidase from Aspergillus niger and the CaMV 35S 15 terminator fragment (see Example 11), into pIC19R (Marsh *et al.*, 1984) linearized with HindIII, resulting in plasmid pMOG440.

20 Plasmid pMOG450 (see Example 6) is digested with HindIII. The HindIII fragment, containing the class-I patatin promoter, the DNA fragment encoding mature  $\alpha$ -amylase from Bacillus licheniformis and the nos terminator from Agrobacterium tumefaciens, is cloned in the binary vector pMOG23 linearized with HindIII. This results in the binary 25 vector pMOG436.

Plasmid pMOG440 is digested with EcoRI. The EcoRI fragment, containing the class-I patatin promoter, the cDNA fragment encoding mature glucoamylase from Aspergillus niger and the CaMV 35S terminator, is cloned in the binary plasmid 30 pMOG436, linearized with EcoRI. Using restriction enzyme analysis, transformants are screened for the presence of the two expression cassettes in a tandem orientation. The binary vector with the expression cassettes having this orientation, called pMOG437 (Figure 6) is used for transformation experiments.

The chimeric  $\alpha$ -amylase gene from Bacillus licheniformis and the chimeric glucoamylase gene from Aspergillus niger, both under the control of the tuber-specific class-I patatin promoter, as present on the binary plasmid pMOG437, are mobilized in a triparental mating with the *E. coli* strain HB101 containing plasmid pRK2013 (Ditta *et al.*, 1980) into Agrobacterium strain LBA4404 which contains a plasmid having the virulence genes necessary for T-DNA transfer to the plant (Hoekema *et al.*, 1983).

Example 11Transformation of potato

Potato (Solanum tuberosum cv. Désirée) was transformed 5 with the Agrobacterium strain LBA4404 (pMOG437) as described by Hoekema *et al.* (1989).

The basic culture medium was a MS30R3-medium, consisting of MS-medium (Murashige & Skoog, 1962), supplemented with 30 g/L sucrose and with R3-vitamins (Ooms *et al.*, 1987) and, 10 where indicated, 5  $\mu$ M zeatin riboside (ZR) and 0.3  $\mu$ M indole acetic acid (IAA). The media were solidified where necessary with 0.7 g/L Daichin agar.

Tubers of Solanum tuberosum cv. Désirée were peeled and surface-sterilized for 20 minutes in 0.6% hypochlorite 15 solution containing 0.1% Tween-20. The potatoes were washed thoroughly in large volumes of sterile water for at least 2 hours. Discs of approximately 2 mm thickness were sliced from cylinders of tuber tissue prepared with a corkscrew. Discs were incubated for 20 minutes in a suspension consisting of 20 the MS30R3-medium without ZR and IAA, containing between  $10^6$ - $10^7$  bacteria/ml of Agrobacterium LBA4404 (pMOG437). The discs were subsequently blotted dry on sterile filter paper and transferred to solid MS30R3-medium with ZR and IAA. Discs were transferred to fresh medium with 100 mg/L cefotaxim and 25 50 mg/L vancomycin after 2 days. A week later, the discs were again transferred to the same medium but this time 100 mg/L kanamycin was present to select for transgenic shoots. After 4-8 weeks, shoots emerged from the discs at a frequency of 5- 10 shoots per 100 discs. Shoots were excised and placed on 30 rooting medium (MS30R3-medium without ZR and IAA, but with 100 mg/L cefotaxim and 100 mg/L kanamycin), and propagated axenically by meristem cuttings and transferred to soil. The plants were allowed to tuberize and were subsequently tested for expression of the genes of interest.

35

Example 12

Simultaneous tuber-specific expression of both  
 $\alpha$ -amylase (*Bacillus licheniformis*) and glucoamylase  
(*Aspergillus niger*) in potato and carbohydrate

analysis of transgenic tubers

Potato plants are transformed with binary vector pMOG437 as described in Example 7. The plants are assayed for both  $\alpha$ -amylase and glucoamylase activity. Alpha-amylase activity is 5 determined as described in Example 4. The presence of glucoamylase is demonstrated by Western blotting, using antibodies raised against Aspergillus niger glucoamylase. Plant material (about 50 mg) is homogenized in 100  $\mu$ l assay 10 buffer and homogenized. The homogenate is spun for 10 minutes in an Eppendorf centrifuge. The supernatant is tested for  $\alpha$ -amylase activity, for the presence of glucoamylase and for protein content. The presence of the enzymes is only detected 15 in the tubers of the transgenic potatoes.

Tubers of transgenic potatoes expressing both enzymes are 15 analyzed for the presence of soluble sugars by HPLC. A higher content of soluble sugars is found in transgenic tubers as compared to control plants.

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Claims

1. A method for modifying the carbohydrate composition of a plant or plant organ characterized by the growing of a 5 transgenic plant containing an expression cassette which contains a DNA sequence encoding a (primary) enzyme of interest capable of degrading a plant polysaccharide, under conditions conducive whereby said enzyme-encoding DNA sequence is expressed and the carbohydrate composition of 10 said plant or plant organ is modified, with the proviso that if said plant or plant organ is potato, the DNA sequence encoding said (primary) enzyme of interest originates from a microbial source.
- 15 2. The method of claim 1 further characterized in that said expression cassette contains a regulatory sequence capable of directing the expression of said enzyme of interest at a selected maturity stage of the development of the transgenic plant or plant organ.
- 20 3. The method of claim 1 further characterized in that said expression construct is capable of directing the tissue-specific expression of said enzyme of interest.
- 25 4. The method of claim 1 further characterized in that the DNA sequence encoding said (primary) enzyme of interest is provided with a leader sequence capable of targeting the expressed enzyme to a pre-determined cellular compartment or organelle.
- 30 5. The method of claim 1 further characterized in that an increase in the content of soluble saccharides containing up to six monosaccharide units is obtained in said transgenic plant or plant organ as a result of the action of said enzyme 35 of interest.
6. The method of claim 1 wherein said (primary) enzyme of interest is selected from the group selected from amylases, glucanases, cellulases, endoglucanases, arabinanases,

galactanases, mannanases, xylanases, fucosidases, rhamnosidases, levanase and inulanase.

7. The method of claim 1 wherein the DNA sequence encoding 5 said (primary) enzyme of interest originates from a microbial source.

8. The method of claim 7 wherein the DNA sequence encoding 10 said (primary) enzyme of interest is selected from the group consisting of an  $\alpha$ -amylase originating from Bacillus licheniformis and a glucoamylase originating from Aspergillus niger.

9. The method according to claim 1 further characterized in 15 that said transgenic plant contains one or more expression constructs containing DNA constructs encoding a secondary enzyme of interest other than and in addition to said (primary) enzyme of interest, said secondary enzyme of interest being capable of using the starch degradation 20 products resulting from the action of said primary enzyme of interest as a substrate.

10. The method of claim 9 further characterized in that said additional enzyme of interest is selected from the group 25 consisting of glucoamylases, pullulanases, isoamylases, cyclomaltodextrin-D-glucotransferases,  $\alpha$ -(1-4)-glucanases,  $\alpha$ -(1-4)-glucosidases,  $\alpha$ -(1-6)-glucosidases,  $\beta$ -glucosidases, D-glucoseisomerase and inulinases.

30 11. The method of any one of claims 1 - 10 further characterized in that said transgenic plant is selected from the group consisting of tomato, potato, corn, cassave, carrot, lettuce, strawberry and tobacco.

35 12. An expression construct characterized in that a DNA sequence encoding an enzyme of interest capable of degrading a plant polysaccharide is operably linked to a regulatory sequence capable of directing the expression of said enzyme of interest at a selected maturity stage of the development

of a transgenic plant or plant organ.

13. An expression construct characterized in that a DNA sequence encoding an enzyme of interest capable of degrading 5 a plant polysaccharide is operably linked to the 35S CaMV promoter.

14. An expression construct characterized in that a DNA sequence encoding an enzyme of interest capable of degrading 10 a plant polysaccharide is operably linked to a regulatory sequence capable of directing the tissue-specific expression of said enzyme of interest.

15. A vector comprising an expression construct according to 15 any of the claims 12 - 14.

16. A transgenic plant characterized in that said plant contains an expression cassette according to any one of claims 12 - 14.

20

17. A bacterial strain characterized in that said bacterial strain contains a vector according to claim 15.

25

18. A transgenic plant or plant organ characterized in that said plant or plant organ contains a modified carbohydrate composition a result of the method according to either one of claims 1 and 9.

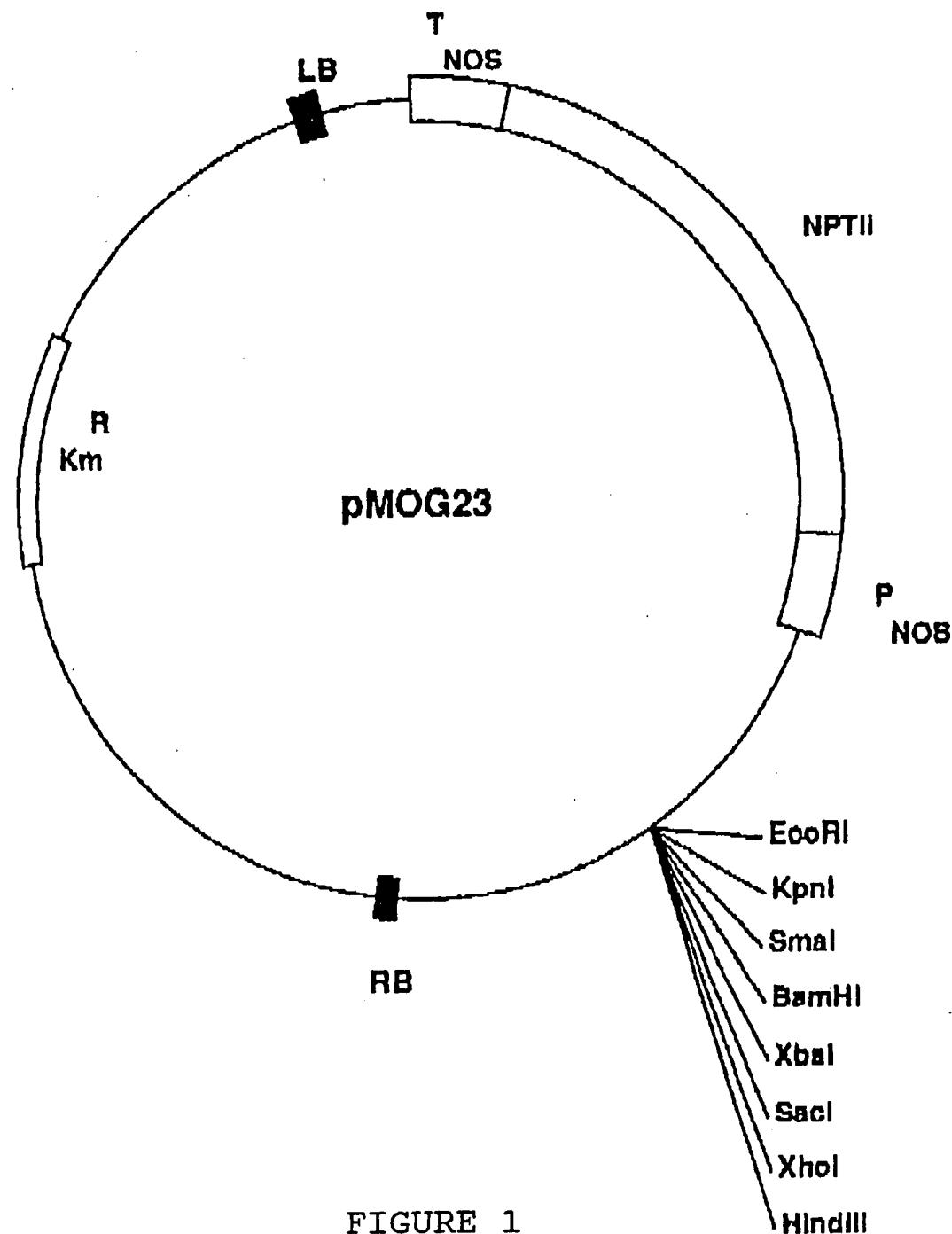


FIGURE 2 (sheet 1 of 2)

<u>Xba</u> I	*	<u>Hpa</u> I
<u>TCTAGAGTC</u>	ATGAAACAAAC	<u>AAAACGGCT</u>
<u>TTACGGCCGA</u>		<u>TTGCTGACGCC</u>
		<u>TGTTTATTTGC</u>
	<u>Pst</u> I	<u>Hpa</u> I
<u>GCTCATCTTC</u>	<u>TGGCTGCC</u> TC	<u>ATTCTGCAGC</u>
		<u>AGGGCGGC</u> A
		<u>AATCTTAATG</u>
		<u>GGACGGCTGAT</u>
<u>GCA</u> GTATT	<u>TAA</u> GTGTACA	<u>TGCCAAT</u> TA
		<u>CGGCCAACAT</u>
		<u>TGGAAGCGT</u> T
		<u>TGCAAAACGA</u>
<u>CTCGGC</u> ATAT	<u>TGGCTG</u> AAC	<u>ACGGTATTAC</u>
		<u>TGCCGTCT</u> GG
		<u>ATTCCCCCGG</u>
		<u>CATAAA</u> GGG
<u>AACGAG</u> CCAA	<u>GCGGAT</u> GTGG	<u>GCTACGGT</u> GC
		<u>TTACGACCT</u> T
		<u>TATGATTAG</u>
		<u>GGGAGTT</u> CA
<u>TCAAA</u> AGGG	<u>ACGGTT</u> CGGA	<u>CAAAGTAC</u> GG
		<u>CACAA</u> AGGA
		<u>GAGCTGCA</u> AT
		<u>CTGCGATCAA</u>
<u>AAGT</u> CTTCAT	<u>TCCC</u> GGGACA	<u>TAA</u> CGTTA
		<u>CGGGGAT</u> GTG
		<u>GTCATCA</u> ACC
		<u>ACAAAGGCGG</u>
<u>CGCTGAT</u> GGC	<u>ACCGAAGAT</u> G	<u>TAACCGCGGT</u>
		<u>TGAAGTCGAT</u>
		<u>CCCGCTGACC</u>
		<u>GCACCCGGCT</u>
<u>AATTTCAGGA</u>	<u>GAACACCTAA</u>	<u>TTAAAGCCTG</u>
		<u>GACACATT</u> T
		<u>CATTTC</u> CGG
		<u>GGCGCGGCAG</u>
<u>CACATACAGC</u>	<u>GATTTAAAT</u>	<u>GGCATTGGT</u> A
		<u>CCATT</u> TTGAC
		<u>GGAACCGATT</u>
		<u>GGGACGAGTC</u>
<u>CCGAAAGCTG</u>	<u>AACCGC</u> ATCT	<u>ATAAGTT</u> CA
		<u>AGGA</u> AAAGGCT
		<u>TGGGAT</u> TTGG
		<u>AA</u> GTTTCCAA
<u>TGAA</u> ACGGG	<u>AACTATG</u> ATT	<u>TG</u> CCGACATC
		<u>GATTATG</u> ACC
		<u>ATCCTG</u> ATG
<u>CGCAGCAGAA</u>	<u>ATTAAGAGAT</u>	<u>GGGGCA</u> CTTG
		<u>GTATGCCA</u> AT
		<u>GAAC</u> TGCAAT
		<u>TG</u> ACGGTT
<u>CCGCT</u> GTGAT	<u>GCTGT</u> CAAAC	<u>ACATTA</u> AAATT
		<u>TTCTT</u> TTTG
		<u>CGGGAT</u> TTGG
		<u>TIAATC</u> ATG
<u>CAGG</u> AAAAA	<u>ACGGG</u> AAAGG	<u>AAATG</u> TTAC
		<u>GGTAG</u> CTGAA
		<u>TATGGC</u> CAGA
		<u>ATGACT</u> TTGG

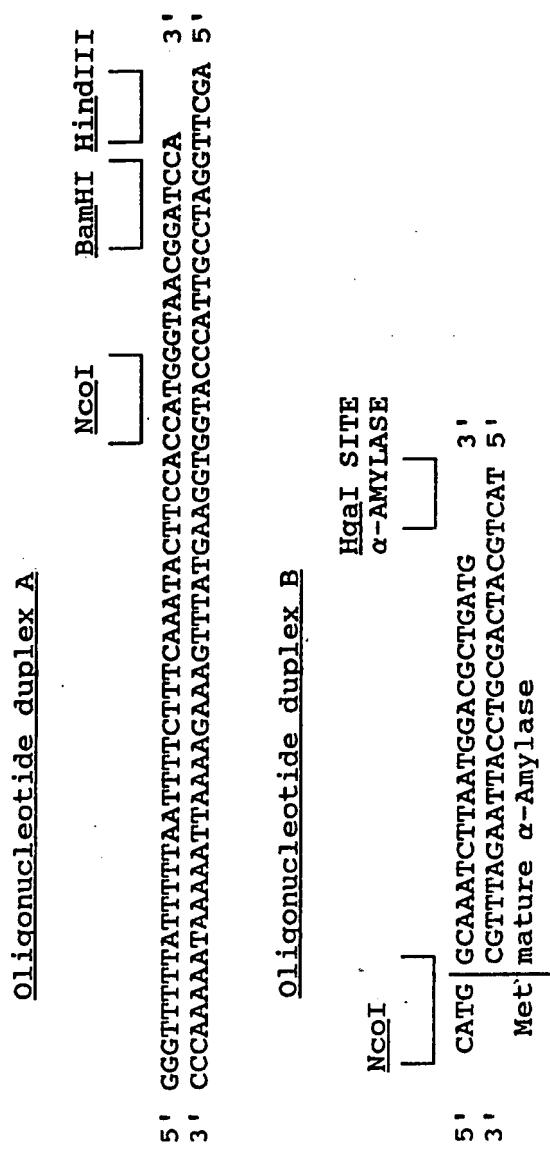
## FIGURE 2 (sheet 2 of 2)

CGCGCTGGAA AACTATTGAA ACAAAACAAA TTTTAATCAT TCAGTGTGTTG ACGTGGCGCT  
 TCAATTATCAG TTCCATGCTG CATCGACACA GGGAGGGGC TATGATATGA GGAATTGCT  
 GAACGGGTACG GTCGTTTCCA AGCATCCGTT GAAATCGTT ACATTTGTCG ATAAACCATGA  
SalI

TACACAGCCG GGGCAATCGC TTGAGTCGAC TGTCCAAACA TGTTTAAGC CGCTTGCTTA  
 CGCTTTTATT CTICAAGGG AATCTGGATA CCCTCAGGTT TTCTACGGGG ATATGTACGG  
 GACGAAAGGA GACTCCCAGC GCGAAATTCC TGCCTTGAAA CACAAATTC AACCGATCTT  
 AAAGCGGAGA AAACAGTATG CGTACGGAGC ACAGCATGAT TATTTCGACC ACCATGACAT  
 TGCGGCTGG ACAAGGGAA GCGACAGCTC GGTTGCAAAT TCAGGTTGG CGGCATTAAAT  
 AACAGACGGA CCCGGTGGG CAAAGCGAAT GTATGTGGC CGGCAAAACG CCGGTGAGAC  
 ATGGCATGAC ATTACCGGAA ACCGTTCGGA GCCGGTTGTC ATCAATTGG AAGGCTGGGG  
 AGAGTTTCAC GTAAACGGCG GGTGGTTTC AATTATGTT CAAAGATAGA AGGGCAGAGA  
BamHI

GGACGGATT CCGAAGGAA ATCCGTTTT TTATTGTCGCC CGTCTTATAA ATTCTTTGA  
 TTACATTTTA TAATTAAATT TAACAAAGTG TCATCAGGCC TCAGGAAGGA CTGCTGACA  
 GTTGAATCG CATAAGTAAG GCGGGATGA AATGGCAACG TTATCTGATG TAGCAAAGAA  
BclI  
 AGCAAATGTG TCGAAATGAA CGGTATCGCG GGTGATCA

FIGURE 3



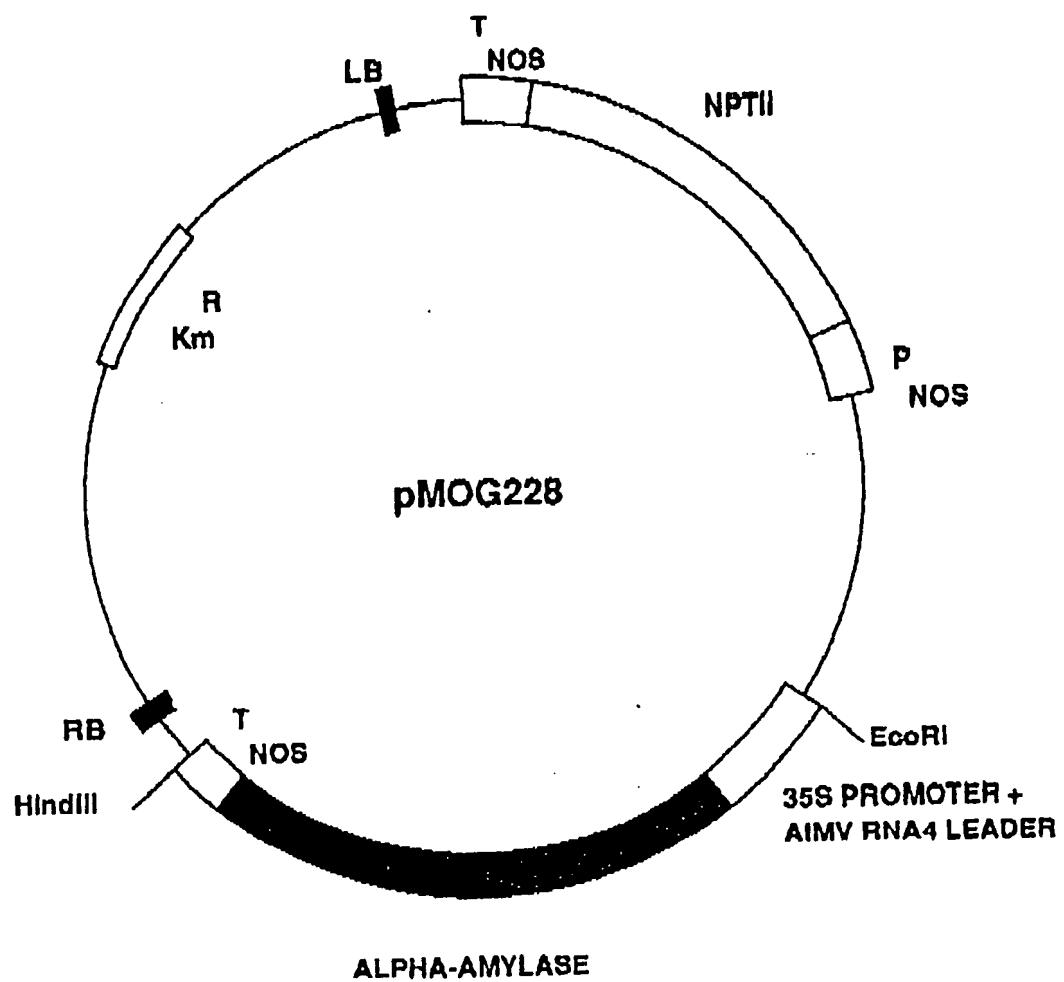


FIGURE 4

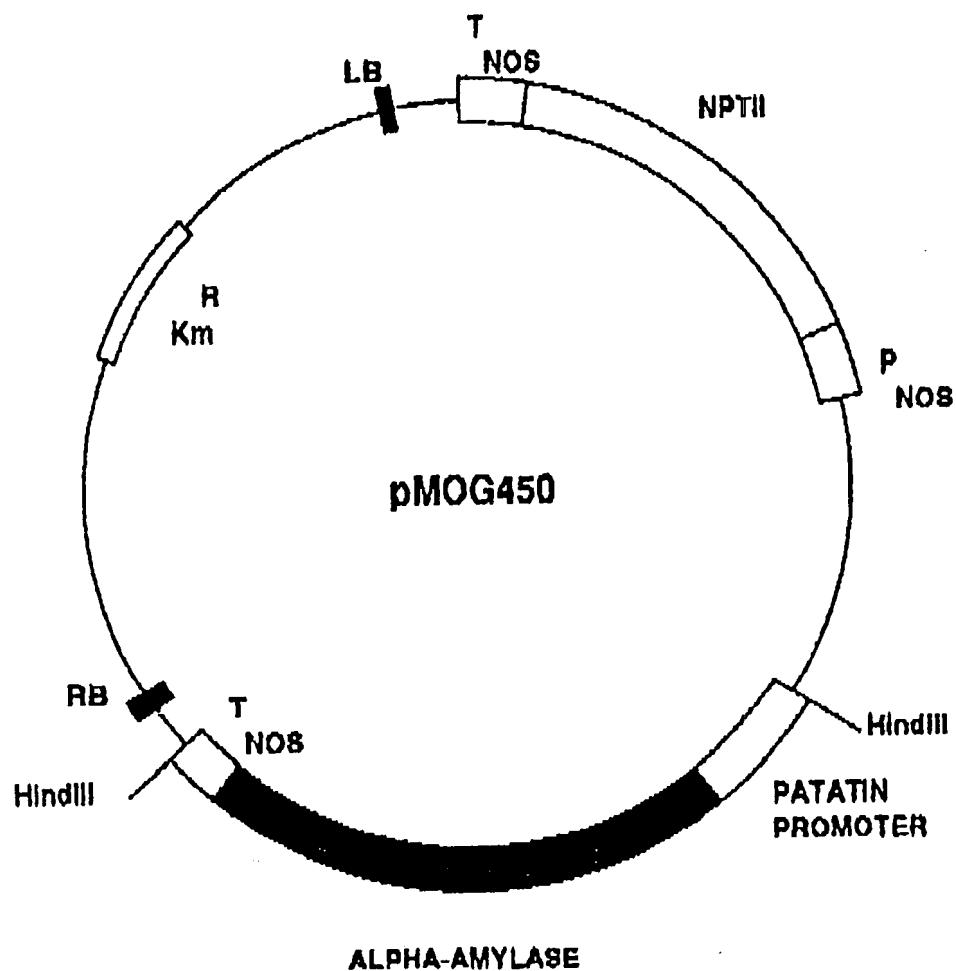


FIGURE 5

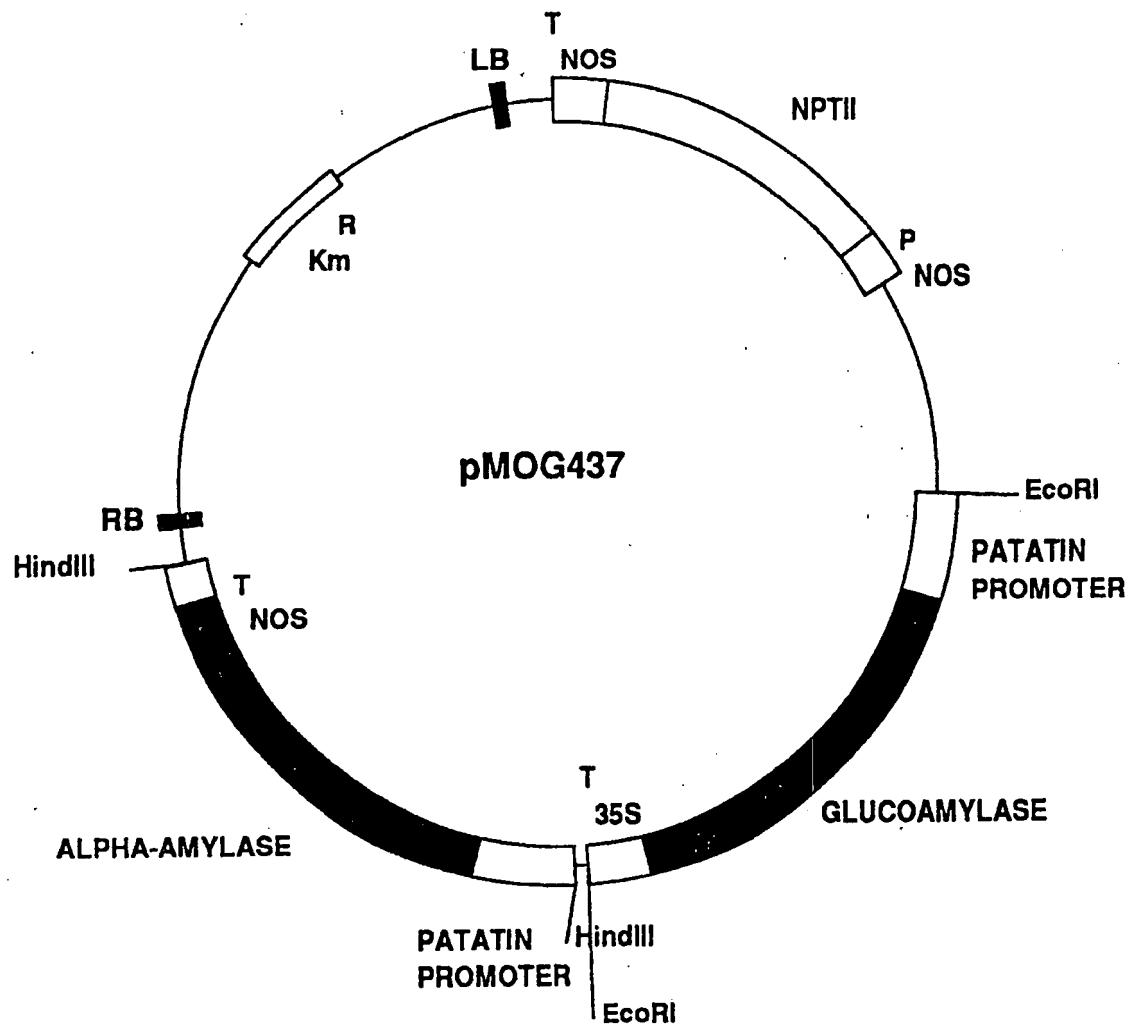


FIGURE 6

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/NL 91/00171

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C12N15/56; C12N15/82; A01H5/00; C12N1/21

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.C1. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	THE PLANT CELL vol. 1, no. 1, January 1989, pages 53 - 63; GIOVANNONI J. J., ET.AL.: 'Expression of a chimeric polygalacturonase gene in transgenic rin <ripening inhibitor> tomato fruit results in polyuronide degradation but not fruit softening' see the whole document ---	1-4, 6, 11, 12, 14-18
X	DD,A,275 704 (AKADEMIE DER WISSENSCHAFTEN DER DDR) 31 January 1990 see the whole document	12, 14, 15
Y	---	1-4, 6-8, 16-18
Y	WO,A,9 001 551 (ROGERS) 22 February 1990 see page 36, line 15 - line 17; example 2 ---	1-4, 6-8, 16-18 -/-

<sup>6</sup> Special categories of cited documents :<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

<sup>7</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>8</sup> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>9</sup> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<sup>10</sup> & document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

1 02 DECEMBER 1991

Date of Mailing of this International Search Report

09. 12. 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

MADDOX A. D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EMBO JOURNAL. vol. 8, no. 2, February 1989, EYNSHAM, OXFORD GB pages 343 - 350; TEERI, T. H., ET AL.: 'Gene fusions to lacZ reveal new expression patterns of chimeric genes in transgenic plants' see the whole document ---	12, 13, 15-18
X	WO,A,8 908 145 (BIOSOURCE GENETICS) 8 September 1989 see examples 11-13 ---	1-3, 7, 11, 12, 15, 16, 18
P, X	WO,A,9 012 876 (AKTIESELSKABET DANSKE SPRITFABRIKKER) 1 November 1990 cited in the application see example 25 ---	12-17
E	WO,A,9 114 772 (MOGEN) 3 October 1991 see examples 14, 15 ---	12, 13, 15-17

**ANNEX TO THE INTERNATIONAL SEARCH REPORT**  
**ON INTERNATIONAL PATENT APPLICATION NO. NL 9100171**  
**SA 51640**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 02/12/91

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
DD-A-275704		None			
WO-A-9001551	22-02-90	AU-A- EP-A-	4037289 0428572	05-03-90 29-05-91	
WO-A-8908145	08-09-89	AU-A- EP-A- JP-T-	4072589 0406267 3502886	22-09-89 09-01-91 04-07-91	
WO-A-9012876	01-11-90	AU-A-	5531890	16-11-90	
WO-A-9114772	03-10-91	EP-A- EP-A- WO-A-	0449375 0449376 9114782	02-10-91 02-10-91 03-10-91	